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Patents ADP number *(if you know it)*
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7482391001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention
DISEASE ASSOCIATED PROTEIN MODIFICATIONS

5. Name of your agent *(if you have one)*
~~AS (3) ABOVE~~

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
Kilburn + Strode
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Description	5
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11. I/We request the grant of a patent on the basis of this application.

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Disease Associated Protein Modifications

The present invention relates to new screening methods to detect disease associated polypeptide modifications and, in particular, to isolate genes encoding such disease associated polypeptides from DNA libraries. The method also relates to screening methods for novel polypeptide interactions which are facilitated by post-translational modifications.

There is considerable interest in "protein display" methods for isolating polypeptides with useful phenotypes from gene libraries encoding a large mixture of polypeptides whereby the corresponding genes can be recovered swiftly and easily. *In vitro* methods of polypeptide display have been developed which allow greater scope for the type of protein displayed than cell based display systems and in particular Ribosome display (patent ref) which facilitates fluid phase separation. However such *in vitro* systems lack many of the biological activities which may be present in cells. This therefore limits the interactions which can be detected to those which do not require post translational modification of the proteins involved and would for example exclude the isolation of modifying enzyme/substrate pairs.

A large number of cellular processes are controlled by the transient interaction between a modifying enzyme and its protein substrate. Such interactions are difficult to detect by conventional methods as the proteins and conditions required for the reaction have first to be identified. In addition the interaction often relies on native protein conformation which is often not achieved during *in vitro* translation. Protein modifying enzymes such as kinases, phosphatases, transferases, proteases etc. control all manner of fundamental cellular processes (Pawson, *Nature*, **373** (1995), pp573) and have also been shown to be involved in disease pathways.

These transient protein/protein interactions can result in a number of different effects some of which can be measured. The kinetic properties of a protein can be altered resulting in altered binding of substrates (Prelich *et al.*, *Nature* **326**, (1989) pp 517) or altered catalysis (Porpaczy *et al.*, *Biochim. Biophys. Acta*, **749** (1983), pp 172). Protein/protein interactions can cause the formation of a new binding site e.g. an ATP binding site is formed by the interaction of the α and βsubunits of the *E. coli* ATPase (Weber *et al.*, *J. Biol. Chem.*, **268**, (1993), pp 6241). Substrate specificity of a protein can be altered by protein/protein interactions as exemplified by the interaction of different transcription factors with RNA polymerase directing the polymerase to specific promoters (White & Jackson, *Trends Genet.*, **8**, (1988), pp 284). Alternatively protein/protein interactions can cause inactivation, for example when a protein interacts with an inhibitor (Vincent & Lazdunski, *Biochemistry*, **11**, (1972), pp2967).

The present invention provides a method for the cell-free cloning of genes encoding disease associated interacting proteins. This method is based upon the reaction of *in vitro* displayed proteins with proteins isolated from a clinical sample. In particular a library of proteins generated from the cDNA from a normal sample is displayed, for example using the technique of Ribosome display, and is incubated with proteins

isolated from a clinical sample. The invention also provides a means for identification of such interactions and rapid isolation of the encoding genes.

Clinical sample refers to a sample which may be tissue, blood or other which could be expected to be affected by the particular disease. The diseased samples refers to a sample which can be demonstrated to be affected by a disease. The normal sample refers to a sample which is not affected by the disease. The normal and diseased samples would ideally be matched to reduce population induced heterogeneity. This may be achieved by obtaining normal and diseased samples from a single patient (e.g. cancerous breast tissue and unaffected breast tissue) or alternatively by pooling diseased and normal samples from a number of different patients.

The generation of cDNA libraries from both normal and diseased samples is performed by standard techniques involving the isolation of mRNA followed by reverse transcription to generate cDNA (Sambrook *et al.*, *Molecular Cloning : a Laboratory manual,* 2nd Ed., Cold Spring Harbor Press, 1989) or alternatively may be achieved using a commercially available kit e.g. PolyATract System (Promega, Southampton, UK). The cloning of the cDNA's into a vector suitable for *in vitro* display by Ribosome display and the conditions for *in vitro* display of the proteins would be as previously described (for example in Hanes and Pluckthun, *Proc. Natl. Acad. Sci.* 94 (1997), p4937). The isolation of total protein from the samples would be achieved by standard methods as detailed Bollag & Edelstein (Protein Methods, Wiley-Leiss, 1991).

It is well documented that post translational modifications such as proteolysis or phosphorylation change the electrophoretic mobility of a protein (Phizicky & Fields *Microbiol. Rev.*, **59**, (1995), pp94). One embodiment of the invention therefore provides a method whereby disease associated protein modifications would be detected using 2D gel electrophoresis. A cDNA library would be constructed from the clinical sample as described. To facilitate subsequent purification of the proteins, a 3' tag such as His or Flag may be incorporated into the transcription vector. This will be achieved by standard molecular biology techniques that will be familiar to those skilled in the art. To facilitate subsequent detection of the proteins, they may be labelled during the translation process with labels such as ^{35}S or biotin. Expression of the proteins would be as previously described. Following translation, the proteins could if required be purified to remove ribosomes and other factors for example by passage of the translation mixture over an affinity matrix containing ligands such as anti-Flag antibodies or nickel where the proteins have 3' tags or using anti-ribosome antibodies to remove ribosomal components onto a solid phase or by simple ultracentrifugation of ribosomes. Total protein from the clinical sample would then be incubated with the purified *in vitro* translated library to facilitate the interaction of the proteins. The total reaction would then be separated by 2D gel electrophoresis (Cash, J. Chromatography 698 (1995), p203) and the resultant gels analysed using appropriate computer software such as Phoretix-2D (Phoretix International, Newcastle upon Tyne, UK). A control reaction would be performed such that the *in vitro* displayed protein library would be reacted with total protein from an alternative clinical sample, for example a normal sample if the first sample is a diseased sample.

Disease associated protein/protein interactions would be identified as unique bands seen with the diseased proteins and not seen in the control proteins.

In order to facilitate rapid identification of the gene associated with protein identified by the 2D gel analysis, the Ribosome displayed library would initially be sub-divided into a number of pools, each pool containing a proportion of the library. This would be achieved by dividing the initial transcription mixture into pools of equal volume. Each pool would then be screened as described above to identify a pool containing a protein of interest. The transcription mix for that pool would then be sub-divided again and the screening process repeated. This procedure would be repeated using progressively smaller pools of ribosome displayed proteins until a single clone was identified which encoded the protein of interest.

Alternatively, following identification of a potential disease associated protein by 2D gel analysis, the protein would be purified from the gel according to standard protocols (Hager & Burgess, *Anal. Biochem.*, **109**, (1980), pp76)and the purified protein panned with a scFv library to identify an antibody(s) which recognise the protein. The antibody(s) would then be used to screen a Ribodisplayed protein library constructed from the cDNA of the diseased sample to identify the protein with its associated gene tag.

In another embodiment of the invention the total protein from a clinical sample would be immobilised onto a solid phase where the solid phase may be a protein chip (Hutchens & Yip, *Rapid Comms. Mass Spec.* **7**, (1993), pp576) or microtiter plate or other solid phase. Immobilisation of the protein could be achieved by either covalent or non-covalent linkage. Non-covalent attachment of the proteins could be facilitated by labelling the proteins for example with biotin using a commercially available reagent such as Sulfo-SBED (Pierce & Warriner, Chester, UK) which could then be reacted with avidin. Covalent attachment of the proteins could be achieved by activating the proteins with reactive species to facilitate cross-linking by any of the conventional ways, such as those described in O'Sullivan *et al.*, (Anal. Biochem. 100 (1979),108). The immobilised proteins would then be incubated with a Ribodisplayed library from a clinical sample. To prevent the degradation of the RNA, RNase inhibitors such as RNasin and vanadyl ribonucleosides would be included in the incubation mixture. Identification of bound Ribodisplayed proteins would be achieved by amplifying their attached mRNA's by PCR.

Yet another embodiment of the invention is based upon the binding of an *in vitro* displayed protein with a protein from a disease sample whereby molecular tags on the proteins can be used as the basis for molecular separation of the interacting protein pairs from the non-interacting proteins. The ribosome-displayed protein may for example be tagged on the RNA for example with an RNA binding protein (such as HIV tat protein) and the proteins from the disease sample tagged with biotin such that consecutive passages of the protein mixture over affinity matrices comprising anti-tag antibodies and avidin would select for binding proteins with the associated mRNA which can be used to determine the gene sequence of one of the binding proteins.

In a similar but alternative embodiment of the invention, the associated mRNA in a protein-ribosome-mRNA complex may be produced with a nucleotide sequence tag originally encoded by the plasmid vector encoding the mRNA. The nucleotide sequence tag may be variable or randomised in the plasmid vector preparation, for example by producing the plasmid vector using a synthetic region encoded by a random oligonucleotide mixture (with appropriate ends which anneal to the vector) to place random a sequence tag at, for example, the 5' end of the mRNA. Following production of the protein-ribosome-mRNA complexes from the gene library, the mRNA sequence tags may then be annealed to an array of synthetic oligonucleotides individually positioned over a solid surface such as a glass slide (a "DNA biochip"). In this manner, individual proteins may finally be positioned at specific locations on the biochip. Upon analysis and identification of proteins which bind or modify the individual proteins on the biochip, the identity of these proteins may subsequently be determined from the known sequence of the immobilised oligonucleotide on the chip whereby this sequence is used to probe the plasmid library (for example, by PCR) in order to identify the specific gene associated with the complementary sequence tag within the plasmid library. The identity of the proteins may alternatively be achieved by PCR amplification of the mRNA sequence associated with the individual protein. This embodiment therefore relates to a protein array biochip with, in principle, individual proteins located at individual loci on the chip.

Overall, the principle of the invention is to provide a readily generated source of proteins generated *in vitro* derived from a clinical sample which are then used to test for protein-protein interactions such as binding and modifications by incubation with clinical samples including either crude samples or samples processed into certain fractions.

The invention is illustrated by but not limited by the following examples.

Example 1

The starting point was a cDNA library constructed from human colon (Clontech, UK). The cDNA,s were recloned into a pGEMT7/SP6 plasmid (Promega, Southampton, UK) providing an upstream promoter for T7 RNA polymerase using long synthetic oligonucleotides and PCR to provide an upstream bacterial ribosome binding site, a downstream spacer derived from the M13 phage gene III and a 3' transcriptional terminator region from the *E. coli* lpp terminator.

In vitro transcription of the pT7 plasmids was performed using a RiboMAX™ kit (Promega, Southampton, UK) according to the manufacturer's instructions. The resultant mRNA was purified according to the manufacturer's protocol.

In vitro translation was performed using *E coli* S30 Extract System with the inclusion of ^{35}S methionine (Promega, Southampton, UK) according to the manufacturer's instructions. The ribosomes were dissociated from the proteins by treatment with EDTA as described by Hanes & Pluckthun (ibid) and the ribosomes removed by centrifugation (100,000 g for 30 min).

A crude protein extract was prepared from a colorectal carcinoma sample (ref). This was then incubated with the *in vitro* translated proteins above for 30 mins at 37°C in the presence of 1 mg/ml vanadyl ribonucleoside complexes and 10 units RNasin. As a control the *in-vitro* translated proteins were incubated with a crude protein preparation from normal colon tissue. The protein mixture was then solubilised according to the protocol of Cash *et al.*, (*Electrophoresis* **18** (1997), p2580). The soluble proteins were then analysed by 2D PAGE (Cash *et al.* ibid) and the proteins detected by autoradiography (Patton *et al.*, *BioTechniques* **8** (1990), p518). Images of the gels were captured using a video camera connected to an image analysis system and then transferred to Phoretix-2D for further analysis. Analysis was performed using Phoretix-2D systems according to the manufacturer's instructions.

A total of 12 protein spots were identified in the in the colorectal cancer reacted proteins that were not present in the control.